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Studying the Expression of a Novel Microsatellite mRNA in Chironomous Riparius

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STUDYING THE EXPRESSION OF A NOVEL MICROSATELLITE mRNA IN *Chironomous riparius*

by

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ABSTRACT

The expression of a unique mRNA containing a microsatellite sequence was studied in *C. riparius* using differential display. First, the microsatellite fragment was sequenced using Sanger's Sequencing method. The microsatellite cDNA contained 11 "ACA" repeats, 12 "AC" repeats, and 5 "CTC" repeats. Second, the affects of cadmium and heat shock on microsatellite mRNA expression were studied. In cadmium experiments, larvae were exposed to low cadmium concentrations (0, 0.04, 0.4, and 4.0 mM) for 24 and 48 h and high cadmium concentrations (0, 1.0, 2.0, 4.0, 8.0, and 16.0 mM) for 48 h. The effects of cadmium on fed larvae at 48 h was also investigated. Results showed that microsatellite mRNA expression in *C. riparius* was unaffected by cadmium at either time point without food ($p = 0.732$, 0.885, 0.702, respectively). Feeding did not significantly alter microsatellite mRNA expression in *C. riparius* ($p = 0.857$). In heat shock experiments, the starved larvae were exposed to 20, 28, 32, and 37 °C at 30 min. Results show that the level of microsatellite mRNA expression was significantly reduced by 37 °C compared to 20 °C ($p = 0.002$). The levels of microsatellite mRNA was significantly increased when the temperature was elevated from 20 to 28 °C ($p = 0.03$). The 32 and 37 °C groups were significantly lower than the 28 °C group ($p = 0.014$ and 0.002, respectively). Previous work in *C. riparius* has shown that ribosomal L8 mRNA expression is consistent at all the temperatures tested (Govinda *et al.*, 2000). Differential display was run using the same mRNA samples as used for the microsatellite mRNA heat shock experiment. Results showed that ribosomal mRNA was consistently expressed in heat shock mRNA at all temperatures tested ($p = 0.173$). In

actinomycin D experiments, a concentration of 96 nM actinomycin D down regulated microsatellite mRNA transcription in *C. riparius* ($p = 0.054$). The following was learned from this project: 1) Results from the heat shock experiment showed that differential display is a sensitive method to detect gene expression. 2) Results from cadmium studies suggested that microsatellite mRNA can be used as a negative control in future cadmium toxicity tests in *C. riparius*. 3) Results from heat shock studies indicated that this type of stress does modify microsatellite mRNA expression in *C. riparius*..

INTRODUCTION

All eukaryotes carry random repeated sequences through out their genome mostly in non-coding regions (Stephan and Soowon, 1993). The functions of these tandem repeated sequences are not completely known (Griffith *et al.*, 1996). Microsatellites, a class of tandem repeated sequences, contain a high degree of polymorphism, thus they can be used for population genetic studies (Rongnoparut *et al.*, 1999). This work describes a unique mRNA containing a microsatellite sequence. Its expression in response to cadmium (Cd) elevated temperatures (heat shock), and a transcription inhibitor (ActD) was investigated.

Based on their repeat lengths and array sizes, the tandem-repetitive sequences are categorized into three groups of satellite, minisatellite, and microsatellite (Levinson and Gutman, 1987). Satellite DNAs are more ordered, and they are the largest of the repeated sequences. "Satellite DNA is found to consist of multiple tandem repeats of short nucleotide sequences, stretching up to hundreds of kilobases in length." (Griffith *et al.*, 1996). Satellite DNAs are located in heterochromatic regions close to centromeres and telomers, where meiotic recombination is very low (Mather, 1939). The large size and ordered structure of satellites are due to their location in the region of low recombination (Charlesworth *et al.*, 1986). In contrast, minisatellites are found in euchromatic regions near subtelomers where the recombination rate is very high (Royle *et al.*, 1988). Minisatellites are also known as VNTRs (variable number tandem repeats). The number of repeated units could be 15-100 base pairs long (Griffith *et al.*, 1996). Minisatellites have variable numbers of repeated sequences at different loci, and they vary from

individual to individual (Griffith *et al.*, 1996). Microsatellites (MS), another class of tandem repeated sequences, are close to minisatellites in terms of size. Like minisatellites, MS have variable numbers of repeated sequences, and they vary from individual to individual. MS are rich in sequences of 'CA' (Griffith *et al.*, 1996). MS consist of repeats of dinucleotide, trinucleotide, or quatronucleotide base pairs. Each repeated unit is usually less than 100 base pairs long. MS are mostly located in non-coding regions. Surprisingly, during the course of this project, we determined that MS is also found in coding regions. "Because their high level of variation makes them a powerful tool for identifying individuals and measuring gene flow, microsatellites have been used in genome mapping, in paternity exclusion, and in population genetic studies" (Michalakis and Veuille, 1996; Colson and Goldstein, 1999). Since MS is used to determine kinship or relations within a population, we wanted to study MS mRNA expressed in *Chironomous riparius* in preparation for future ecological studies.

Why chiromonids? Chironomids are the larvae of midge fly. They are found in freshwater and estuaries. Chironomids are the most abundant and the most widely distributed groups of insects worldwide (Armitage *et al.*, 1997). There are 15,000 different species of chironomids (Armitage *et al.*, 1997). Chironomids spend most of their life cycle (the larval stage) in water and sediments. The life cycle of chironomids starts by the adult female laying eggs on water. Subsequently, the larvae hatch and live in sediments. After four instars, larvae pupate. The pupae swim to the surface, and the young adults emerge leaving the water (Pinder, 1986). Chironomids are a very good source of food for fish, invertebrates, and some vertebrates living in water. The larval stage is crucial in an ecological context (Oliver, 1971) as well as to study freshwater

toxicology (McCahon and Pascoe, 1988a). Chironomids are the most dominant species found in polluted ponds, lakes, and rivers (Yamamura *et al.*, 1983). They are an EPA approved sediment test organism (US EPA, 1996) and can be used to measure water quality.

Pollution caused by heavy metals, either natural or anthropomorphic, has been a major concern and problem in aquatic ecosystems (Kaviraj and Konar, 1982). Natural sources of heavy metal contamination are volcanic activities and forest fires (Nriagu, 1977). Sources of anthropomorphic pollution are industries such as mining and manufacturing, automotive combustion, sewage, and agriculture chemicals (Nriagu, 1977; Nriagu and Pacyna, 1988). Examples of heavy metals that contaminate water and sediment are cadmium (Cd), copper, silver, mercury, lead, and zinc (Sanders *et al.*, 1993; Al-Madfa *et al.*, 1994). Research scientists are working hard to develop new techniques that can be used to measure such contamination and develop bioassays that are crucial in establishing good water quality criteria (Khangart and Ray, 1989).

Aquatic organisms resist toxicants by overexpressing or downregulating certain genes. A good example of a heavy metal detoxification mechanism is metallothionein (MT), which is also known as a metal-binding protein. Margoshes and Vallees first isolated MT in 1957 from horse kidney. It was called the Cd-binding protein. MTs are a small protein rich in cysteine (Fowler *et al.*, 1987). Further research proved that MT could be induced by Cd in a number of species including mammals (Kagi & Nordberg, 1979), fish (Noel-Lambot *et al.*, 1976), limpets (Howard & Nickless, 1977) and crabs (Olafson *et al.*, 1979). These animals build tolerance to metal exposure by over expressing MT (Yamamura *et al.*, 1983). MT is widely distributed and found in

prokaryotes, protists, fungi, plants, as well as animals (Higham *et al.*, 1986; Kagi & Kojima, 1987).

Heat shock proteins, another class of stress proteins, are induced in response to a variety of stressors such as heat (Bauman *et al.*, 1993), ethanol (Li, 1983), transition series metals (Burdon *et al.*, 1982; Levinson *et al.*, 1980), sodium arsenite (Burden *et al.*, 1982), thiol reagents (Burden, 1986), and amino acids (Thomas & Mathews, 1989). Since heat shock proteins can be induced by many agents, it's difficult to know the exact mechanism responsible for induction (Burdon, 1986). Heat shock proteins are used by the organisms to provide protection from environmentally induced cellular damage (Sanders, 1993). Ritossa (1962) first isolated heat shock proteins. He reported dramatic changes in gene activity of *Drosophila hydei* larvae by observing changes in "puffing" patterns in the salivary gland polytene chromosomes. Heat shock proteins are also referred to as stress proteins (Sanders, 1993). Heat shock proteins are very conserved and found in many different types of organisms such as bacteria, molluscs, and humans (Schlesinger, 1986; Margulis *et al.*, 1989). When *Brachionus plicatlis* (rotifer) is exposed to different concentrations of copper sulfate, an increase in level of stress protein is observed (Cochrane *et al.*, 1991). In our laboratory, we have used Cd as a chemical specific stressor and heat shock as a general stressor in gene expression studies in *C. riparius*.

To study the effects of environmental stressors such as Cd and heat shock on MS mRNA expression in *C. riparius*, differential display (DD) was used. DD is a DNA fingerprinting method developed by Pardee and Liang in 1992 that is used to study and identify differentially expressed genes. In this method, mRNAs are isolated and

subjected to reverse transcription using an oligo dT primer, which binds the poly A tail of mRNAs. Subsequently, the product of reverse transcription (cDNA) is subject to PCR, using the same oligo dT primer plus a non-specific forward primer. Finally, the products are separated on a sequencing gel and visualized by autoradiography. The bands can be sequenced and/or cloned for identification of known genes or novel genes. In this project, primers specific for MS mRNA were used instead of random primers.

This study was pursued because MS mRNA was identified in Cd-induced larvae of *C. riparius*. Based on this observation, we hypothesized that MS might respond to stressors. Our primary objective was to study the expression of MS mRNA in *C. riparius* exposed to different concentrations of Cd. Thus, we could determine whether or not MS was a stress responsive gene. As far as we know, there have been no reports on MS containing mRNAs or their induction by stressors. Another reason for pursuing this mRNA was that it might be used in future studies to measure genetic diversity in populations of chironomids. This was based on the literature described earlier, in which MS has been used to determine genetic diversity in other organisms. Studies have shown that pollution can play a crucial role in natural selection among aquatic organisms (Postma *et al.*, 1994). The expression of MS mRNA was studied by experiments involving the exposure of *C. riparius* to different stressors such as Cd and heat shock. The ability of DD to measure MS mRNA expression was also evaluated.

MATERIALS AND METHODS

A. Animals

The test organism was 4th instar larvae of *C. riparius*. *Chironomus* larvae (*Chironomus riparius*) were a gift from Dr. Alan McIntosh, University of Vermont. The larvae were maintained at 20 ± 1 °C [room temperature (RT)] with a 12:12 hours light:dark photoperiod. The *Chironomus* larvae were raised on a substrate consisting of acid-washed sand and cerophyll. Water for cultures and experiments was particle and carbon filtered [CDPRM1206 and CDFC01204, respectively (Millipore Corp., MA)] and allowed to-stand 48 hours in order to remove residual chlorine. It had a pH of 7.3 (Sentron Model 2001 pH System, Sentron Inc., WA) and hardness of 136 mg/L (Model PHT-CM-DR-LT, Ward's Natural Science Establishments, Inc., USA). Acid-washed sand was prepared by placing sterilized, natural play sand (American Stone Mix, Inc., MD) in I0 percent HCl for 3 - 4 hours, washing it copiously in deionized water and oven drying it overnight. Cerophyll is a dehydrated cereal of grass leaves used by larvae to construct their tubes (Ward's Natural Science Establishment, Inc., NY). Larvae were fed twice a week with a suspension of ground TetraDoro Green® Floating Food Sticks (Tetra, Germany).

B. Total RNA Extraction and Preparation for Differential Display

Total cellular RNA was isolated using TriReagent (Sigma BioSciences, St. Louis, MO). First, 10-12, 4th instar larvae of *C. riparius* were homogenized in 1 ml of TriReagent using a 2 ml glass mortal and pestle. The homogenate was centrifuged at 12,000 rpm and 4 °C for 15 min. Then the supernatant containing DNA and RNA

and protein was transferred into an Eppendorf tube. Second, 0.2 ml of chloroform was added, and the mixture was allowed to stand for 15 min at room temperature (RT). After centrifuging as above, the RNA in the upper layer was transferred to a clean Eppendorf tube. The RNA pellet was formed by precipitating with 0.5 ml of cold isopropanol followed by 10 min of centrifugation as above. The RNA pellet was washed with 0.5 ml of 75% ethanol. The supernatant was removed, and the RNA pellet dissolved in 100 µl of pretreated with diethyl pyrocarbonate (depc-water) (Sigma Chemicals, Co., St. Louis, MO). Total RNA was then subjected to Message Clean (GenHunter Corporation, Nashville-TN). The purpose of message clean was to get rid of any DNA contamination. The following steps were performed for Message Clean. First, total RNA was subjected to DNase I digestion. This step ensured digestion of any traces of DNA present in the RNA. Here, 50 µl of total RNA (containing 20 µg) was added to 5.7 µl of 10X reaction buffer and 1.0 µl of DNase I (10 U/µl) followed by incubation for 30 min at 37 °C. Second, samples were extracted with 40 µl of phenol/chloroform (3:1) to remove Dnase I. The samples were vortexed for 30 seconds and allowed to stand on ice for 10 min. Then the mixture was centrifuged for 5 min at 4 °C and 12,000 rpm. Subsequently, the upper aqueous phase was collected into another clean Eppendorf tube. Third, clean total RNA was precipitated using 5 µl of 3 M sodium acetate and 200 µl of 100 % ethanol. The samples were allowed to stand at - 70 °C for more than 60 min. Next, the samples were centrifuged for 10 min at 4 °C and 12,000 rpm. The supernatant was discarded, and the RNA pellet was washed using 0.5 ml of 70 % ethanol by centrifugation as above. The supernatant was then discarded, and the RNA pellet was

dissolved in 10 - 15 µl of depc-water. The RNA concentration was determined by making a 5:995 dilution of the cleaned RNA followed by measuring the absorbance at 260 nm. RNA samples were stored at - 70 °C.

C. Sequencing

MS specific primers were used to generate genomic DNA and cDNA. The sequence for the positive primer (MSP5) was -AGCCCATACCTACCTGTGG and the sequence for the negative primer (MSM25) was - GGCATACAAGAGCAGCTATTGC. The PCR products were run on a 1.5 % agarose gel. The major PCR products were cut out, and the DNA was recovered. Sequencing was performed using the Ampli - Taq Cycle Sequencing kit (Life Technologies, Gaithersburg, MD), which is based on the Sangers Sequencing method (Sanger and Coulson, 1975). Bands obtained by DD were visualized by randomly incorporating $\alpha[^{32}P]$dATP. The sequencing products were separated on a 6 % polyacrylamide gel by electrophoresis using a SequinGen II apparatus (BioRad, Hercules, CA). The dried gel was exposed to X-ray film (Wolf Scientific Autoradiography, Wolf X-ray Corp., W. Hempstead, NY) for 24 h and developed manually. In addition, 3 major bands generated by DD were cut out and sequenced as above.

D. Experimental Design

This project was divided into three major experimental sections. The first part involved Cd experiments. The second part consisted of heat shock experiments. In the third part, ActD experiments were performed.

Part I. Cd Experiments

In this part, the larvae were exposed to different concentrations of Cd. The Cd experiments were further divided into fed versus starved larvae. Experiments involving starved larvae were as follows: 0, 1, and 2 mM Cd for 24 h; 0.0, 0.04, 0.4, and 4.0 mM of Cd for 24 hand 48 h; and 0, 1, 2, 4, 8, and 16 mM Cd for48 h. Experiments involving fed larvae were as follows: 0, I, 2, 4, 8, and 16 mM Cd for 48 h. All of the above experiments were run in duplicate or triplicate. The experimental system consisted of 60 g of acid washed sand and 250 ml of test water prepared as above. Total RNA was isolated from larvae as described above and subjected to DD using primers specific for microsatellite mRNA. See Table 1 in the Results Section for primer information.

Part II. Heat Shock Experiments

Starved larvae were exposed to 20, 28, 32, and 37 °C for 30 min. Experiments were run in triplicate. The test system consisted of 60 ml of test water in a 150 ml beaker. The beaker was placed in a waterbath and brought to appropriate temperature before larvae were added. Total RNA was isolated from larvae as described above and subjected to DD using primers specific for microsatellite mRNA.

Part III. ActD Experiments

Starved larvae were exposed to 0, 8.0, 24.0, 48.0, and 96.0 nM of ActD for 24 h. Experiments were run in triplicate. Total RNA was isolated from larvae as described above and subjected to DD using primers specific for microsatellite mRNA.

Summary of Exposure Conditions:

Treatment	Exposure time	Concentrations or Temperatures
Cadmium (Starved)	24h	0, 1, and 2.0 (mM)
	24/48 h	0, .04, .4, and 4.0 (mM)
	48 h	0, 1, 2, 4, 8, and 16 (mM)
Cadmium (Fed)	48 h	0, 1, 2, 4, 8, and 16 (mM)
Heat shock (Starved)	30min	20, 28, 32, and 37°C
Actinomycin D (Starved)	24h	0, 8.0, 24.0, 48.0, and 96.0 (nM)

E. Differential Display

Differential display (DD) was carried out using an RNA Image kit from GenHunter Corporation (Nashville, TN). DD is a new DNA fingerprinting technique developed by Pen Liang and Arthur B. Pardee in 1992. Our procedure involved subjecting purified RNA to reverse transcription using MSM25, a primer specific for our microsatellite mRNA fragment. The product of reverse transcription was single stranded DNA that was complementary to mRNA. The reaction for reverse transcription was as follows:

RNA was diluted to 0.1 µg/µl using depc-water.

Depc-water	9.4 µl
5X RT buffer (tris buffer pH = 7.8)	4.0 µl
dNTP (250 µM)	1.6 µl
Total RNA	2.0 µl (0.1 µg/µl)
MSM25 - Primer (20pM)	
Total Volume	19 µl

Reverse transcription was carried out in a Gene Amp PCR System 2400 thermocycler (Perken Elmer, Branchburg, NJ). Samples were heated for 5 min at 65 °C, 60 min at 37 °C, 5 min at 75 °C, and stored at 4 °C. After 10 min at 37 °C, the thermocycler was paused and the reverse transcriptase (MMLV) was added; samples then finished the 60 min incubation at 37 °C.

Next, the products from reverse transcription were subjected to PCR (Polymerase Chain Reaction). The PCR kit was obtained from Life Technologies (Gaithersburg, MD). The PCR reaction for DD consisted of the following:

Deionized water	11.8 µl
10X PCR buffer	2.0 µl
dNTP (25 µM)	1.6 µl
MSP5 (20 pmol)	1.0 µl
MSM25 (20 pmol)	1.0 µl
Reverse transcription mix	2.0 µl
[32P]dATP (3000 Ci/mmol)	0.2 µl
Taq DNA polymerase (5 U/ul)	0.2 µl
Total Volume	20.0 µl

PCR conditions were as follows: 94 °C for 3 min, then 35 cycles of 94 °C for 30 sec → 57 °C for 2 min→ 75 °C for 30 sec, followed by heating at 75 °C for 3 min, and storage at 4 °C. The PCR products were separated by polyacrylamide gel electrophresis (PAGE) and visualized as described for DD.

F. Quantification of MS mRNA

Bands from PAGE were analyzed by densitometry using SigmaGel lanes (Sigma Chemical Company, MS). Background was subtracted from total area. Statistical analysis was done by one way ANOVA followed by Tukey Post-Hoc Test, where if $p \leq 0.05$, the results were considered significant.

RESULTS

A. Sequence of MS mRNA

Prior to studying gene expression, the genomic and cDNA bands were sequenced. Total RNA was subjected to reverse transcription using the MSM25 primer. Then, the cDNA products were amplified using MSM25 and MSP5 primers (Table 1). Genomic DNA was amplified at the same time as the cDNA using the same primers. Both genomic and cDNA products were run on a 1.5 % agarose gel (Figure 1). On the gel, lane 1 was the 100 base pairs (bp) ladder, lane 2 was the genomic band, and lane 3 was the cDNA band. The genomic band was slightly bigger(~290 bp) than the cDNA MS band (~260 bp). Both bands were isolated from the gel and sequenced using the Sanger Sequencing method. Both sequences contained the MS mRNA previously found in Cd induced larvae of *C. riparius*. Based on the sequencing information, the genomic band apparently contained a piece of intron at 5' end of the MS gene. The sequencing information indicated that the MS mRNA consisted of 13 "ACA" repeated codons, 5 "CTC" repeated codons, and 12 "AC" repeated base pairs (Figure 2). It was 168 bp in length.

B. Cadmium experiments using differential display

This part of the study involved experiments with Cd in starved or fed larvae using differential display as a technique to measure gene expression. The first study was conducted on starved larvae with Cd concentrations of 0, 1, and 2 mM at 24 h (Figure 3). Samples were run in triplicate. Results indicated that the MS primers were binding to three major bands. After sequencing, band A was identified as ribosomal mRNA, band B was unknown, and band C was identified as MS mRNA. Band C was sequenced and found to be shorter than the cDNA above, 168 bp. This suggested that our forward primer was binding

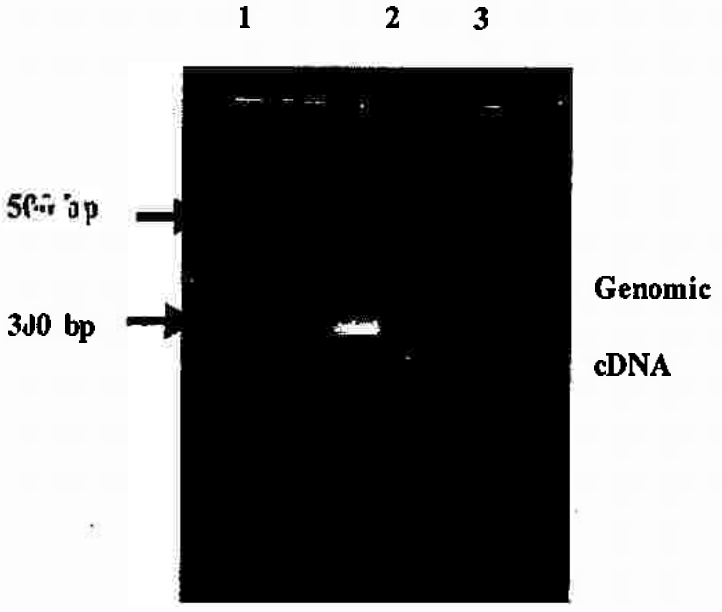

Figure 1. PCR products generated from genomic and cDNA of MS gene from *C. riparius*.

PCR products were separated by 1.5 % agarose gel. Lane 1 was 100 bp ladder, lane 2 was the PCR product obtained from genomic DNA, and lane 3 was the cDNA obtained from MS mRNA. The genomic band was ~290 bp and the cDNA band was ~ 260 bp. The primers were MSP5 and MSM25.

5'-
CTAAAT*AGCCCATACCTACCTGTGG*ATGTGTCAAACCACATCTACGACTAGCT
CAACAACTACCACTAGCTCGACAACAACAACCTCAACCACAACAACCCCTT
CAACAACAACAACAACAACAACTACCAATTGTCCTCCTCCACCTTGTTG*CAA*
TAGCTGCTCTTGTATGCC-3'

Figure 2. Sequence of cDNA MS gene

Primers are underlined and italized. The negative primer sequence is reversed and complementary. Bold letters indicate the repeats of "ACA", "CTC", and "AC" base pairs. The sequence contains, 13 repeats of "ACA", 5 "CTC", 12 "AC" and 16 "CAA" base pairs. This sequence was obtained from Band C in Figure 3. It is smaller than the band in Figure 1, lane 3, which contained the MS sequence.

Table 1. Primer pairs with *Tm values and location on Sequence

Primers	Primer Sequence (5' → 3')	Tm*	Location in Figure 2
MSMP5	AGCCCATACCTACCTGTGG	50	6-24
MSM25	GGCATACAAGAGCAGCTATTGC	54	149 -170

*Tm is the temperature at which half of the primer population is annealed, which was determined by PC Gene (IntelliGenetics, Mountainview, CA). After a series of experiments, it was determined that an annealing temperature of 57 °C was more specific.

Figure 3. Effect of Cd (mM) on RNA expression at 24 h.

The results obtained from DD showed that the MS primers generated three bands. After sequencing bands A and C, band A was determined to be a ribosomal RNA, and band C was identified as MS mRNA. Band B could not be identified. The study focused on MS mRNA expression (band C). This result also showed an induction of MS mRNA at 1.0 and 2.0 mM Cd. All of the samples were run in triplicate.

to more than one site on the MS gene. The remainder of the project focused on the MS mRNA band only. Also, the resnlts from Figure 3 suggested that the MS mRNA bands was induced at 1 and 2 mM of Cd. That was why we continued with the project.

The next step was to use low Cd concentrations (0.0, 0.04, 0.4, and 4.0 mM) in starved larvae to study MS mRNA expression using DD. First, larvae were exposed to low Cd concentrations at 24 h (Figure 4). Results indicated that the level of MS mRNA expression was not affected at these concentrations. The samples were run in duplicates, $p = 0.732$. The mortality rate was low. There was 15 % mortality rate at the highest concentration, 4.0 mM Cd (Table 2). Second, the larvae were given low Cd concentrations but the exposure period was increased to 48 h (Figure 5). This result suggested that increasing time did not have any effects on level of expression. The samples were run in duplicates, $p = 0.885$. However, the mortality rate increased by one fold (Table 2). Larval mortality rate was 30 % at the highest concentration, 4.0 mM Cd. Third, larvae were exposed to high Cd concentrations (0, 1, 2, 4, 8, and 16 mM) at 48 h (Figure 6). The level of MS mRNA expression was not affected at high Cd concentration. The MS mRNA expression was very consistent. The samples were run in duplicate, $p = 0.702$. Larval mortality rate was very high; 65% of larvae died at the highest concentration of Cd (16.0 mM) at 48 h (Table 2).

Finally, the larvae were fed and exposed to high Cd concentrations (Figure 7). The purpose of this study was to see if feeding was important for toxicity. Feeding did not affect MS mRNA expression significantly. Samples were run in duplicates, $p = 0.857$. Larval mortality rate was high; 55% of larvae died at the highest concentration of Cd (16.0 mM) at 48 h (Table 2).

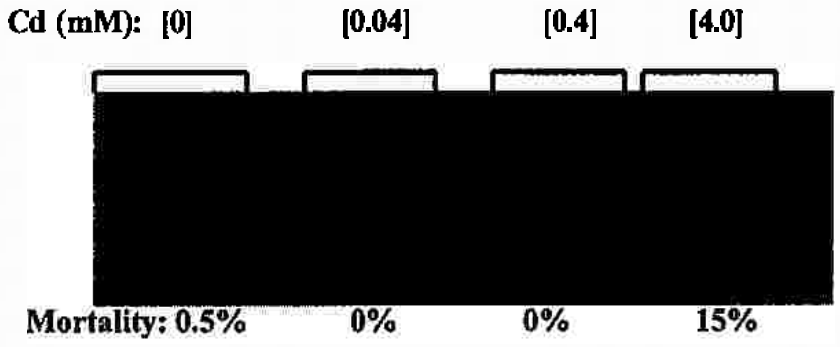

Figure 4. Effects of low Cd (mM) on MS mRNA expression at 24 h.

The results indicated that the expression of MS mRNA was not affected at 0, 0.04, 0.4, and 4.0 mM of Cd at 24 h, $p = 0.732$. Larval mortality was negligible, ranging from 0to 15%. Samples were run in duplicate.

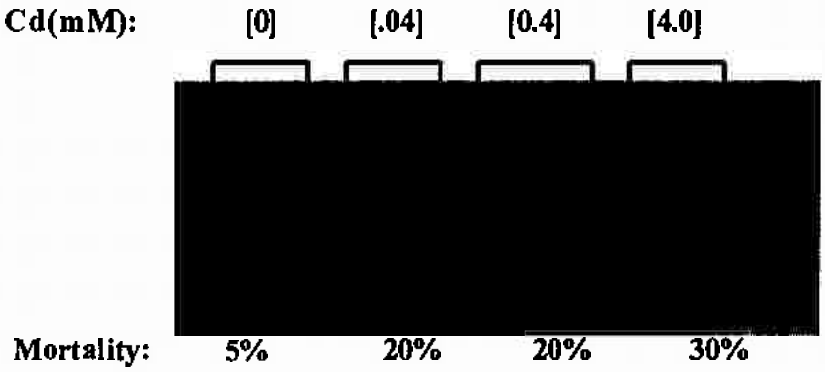

Figure 5. Effects of low Cd (mM) on MS mRNA expression at 48 h.

The level of MS mRNA expression were not significantly altered at 0, 0.04, 0.4, and 4.0 mM of Cd at 48 h, $p = 0.885$. However, larval mortality increased by one fold compare to that at 24 h. Samples were run in duplicate.

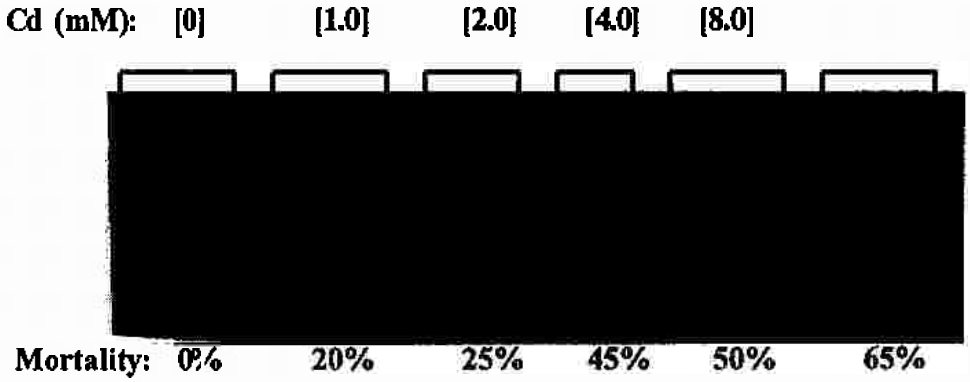

Figure 6. Effects of high Cd (mM) on MS mRNA expression at 48 h.

The levels of MS mRNA expression were not significantly altered by 0, 1.0, 2.0, 4.0, 8.0, and 16.0 mM of Cd at 48 h, $p = 0.702$. However, larval mortality rate increased drastically compared to low concentrations at 24 and 48 h. Samples were run in duplicate.

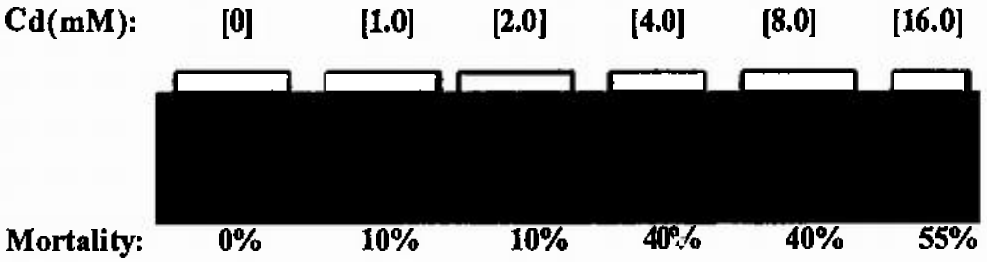

Figure 7. Effects of high Cd (mM) on MS mRNA expression in fed larvae at 48 h.

Statistical analysis showed that there was no difference in the levels of MS mRNA expression at high Cd concentration with feeding, $p = 0.857$. The exposure period was 48 h, and the larvae were fed with a fish food suspension during Cd exposure. Samples were run in duplicate.

Table 2. MS mRNA expression (area) and mortality (%) in different Cd experiments at 24 and 48 h.

Low Cd at24 h (starved larvae)			Low Cd at48 h (starved larvae)			High Cd at 48 h (starved larvae)			High Cd at 48 h (fed larvae)		
Cd (mM)	mRNA (Area)	M (%)	Cd (mM)	mRNA (Area)	M (%)	Cd (mM)	mRNA (Area)	M (%)	Cd (mM)	mRNA (Area)	M (%)
0	2805		0	601		0	1981		0	1310	
0	2891	5	0	688	5	0	1421	0	0	1236	0
0.04	2918		0.04	779		1	1922		1	1342	
0.04	2776	0	0.04	856	20	1	1994	20	1	1343	10
0.4	2289		0.4	1016		2	1878		2	1171	
0.4	2987	0	0.4	400	20	2	1877	25	2	1322	10
4	2590		4	627		4	1761		4	1507	
4	2684	15	4	757	30	4	1961	45	4	1232	40
						8	1969		8	1528	
						8	1930	50	8	445	40
						16	1889		16	1193	
						16	1983	65	16	1157	55

Area = the area determined by "lanes" using SigmaGel

% M = Percent Larval Mortality

n = 10 for each sample

C. Heat shock experiment using differential display

The second part of the project involved heat shock experiments using differential display. The larvae were exposed to 20 °C, 28 °C, 32 °C, and 37 °C for 30 min (Figures 8 and 9). Samples were run in triplicate, $p = 0.002$. The results showed that there was a significant reduction in the level of MS mRNA expression when exposed to 37 °C but not the lower temperatures. See Table 3 for larval mortality rate and band intensity (area) determine by the SigmaGel.

To ensure that the heat shock study was accurate, the heat shock RNA was tested with ribosomal primers. Previous work (Govinda *et al.*, 2000) has shown that ribosomal protein mRNA does not respond to heat shock or Cd. Results showed that the ribosomal protein mRNA expression was very consistent, indicating that the reduction in MS mRNA was real (Figure 10). Samples were run in triplicate, $p = 0.173$. See Table 3 for larval mortality rate and band intensity (area) determine by the SigmaGel.

D. ActinomycinD experiment using differential display

Larvae were exposed to different concentrations of ActD (0, 8, 24, 48, and 96 nM) at 24 h. ActD was used to ensure that the expression of the MS mRNA could be modulated. This was another assay control. Results indicated that there was a significant difference between the control group (no ActD added) and the highest concentration of ActD, 96 nM (Figures 11 and 12). Samples were run in triplicate, $p = 0.054$. Larval mortality and band intensity (area) determined by the Sigam Gel are reported in Table 4.

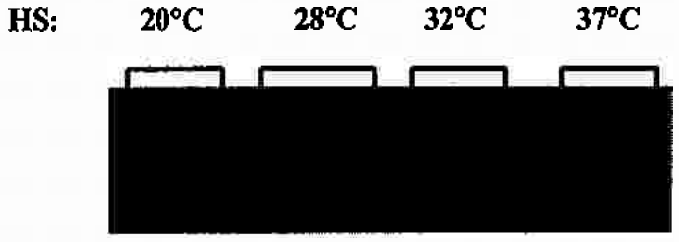

Figure 8. Effects of heat shock on MS mRNA expression at 30 min.

The levels of MS mRNA expression were significantly affected when exposed to different temperatures for 30 min, $p = 0.002$. At 28 °C, the levels of MS mRNA were increased, when compared to room temperature (20 °C), $p = 0.033$. The expression of MS mRNA was significantly reduced at 32 °C and 37 °C, $p = 0.014$ and 0.002, respectively, when compared to 28 °C. Samples were run in triplicate.

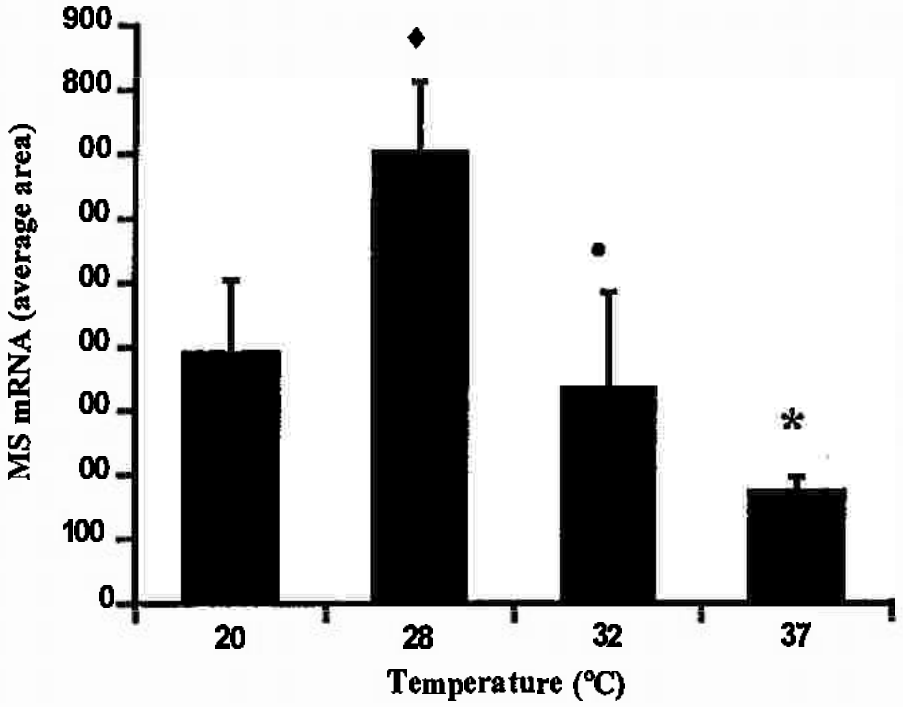

Figure 9. MS mRNA vs. Temperature.

MS mRNA was significantly affected by elevated temperatures, p = 0.002. The level of MS mRNAexpression was induced at 28 °C (♦p=0.003), but reduced at 32 °C (•p=0.014) and 37 °C (*p=0.002) compared to 28 °C. Error Bar= S.D.

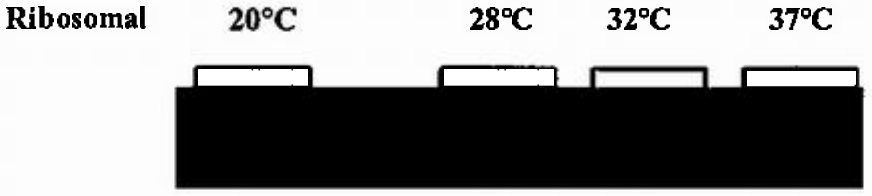

Figure 10. Effects of heat shock on ribosomal protein mRNA expression at 30 min.

The levels of ribosomal protein mRNA expression were not affected by heat shock, $p = 0.173$. This was a control for MS mRNA expression in the heat shock experiment. Samples were run in triplicate.

Table 3. MS mRNA expression (area) and mortality(%) in beat shock Experiment at 30 min.

Temperature (°C)	MSmRNA (Area)	Ribosomal mRNA (Area)	Mortality (%)
20	264	647	0
20	445	462	0
20	469	588	0
28	635	529	0
28	645	557	0
28	829	671	0
32	503	670	0
32	288	545	0
32	217	682	0
37	195	387	0
37	176	496	0
37	153	529	0

Area = the area determine by "lanes" using Sigma Gel

n = 10 for each sample

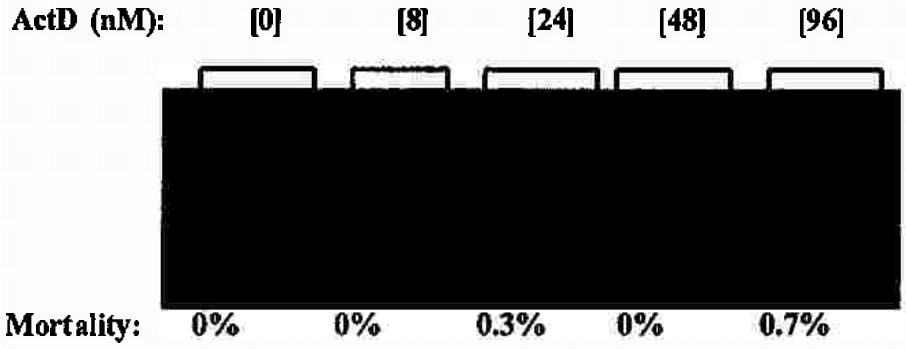

Figure 11. Effects of Actinomycin (nM) on MS mRNA expression at 24 h.

The levels of MS mRNA expression were significantly reduced at the highest concentration of ActD (96 nM), $p = 0.054$. Larval mortality rate was low at 24 h, ranging from 0 to 0.7%. Samples were run in triplicate.

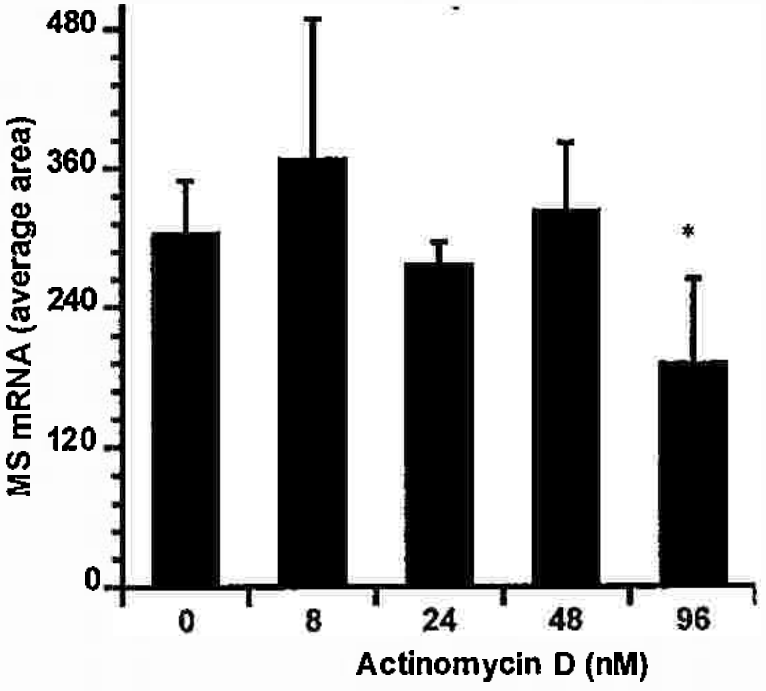

Figure 12. MS mRNA vs. ActD.

The level of MS mRNA was significantly reduced at 96 nM ActD compared to control, * $p = 0.054$. Error Bar= S.D.

Table 4. MS mRNA expression (area) and mortality (%) in ActD experiment at 24 h.

ActD (nM)	MSmRNA (Area)	Mortality (%)
0	261	0
0	297	0
0	352	0
8	504	0
8	272	0
8	327	0
24	268	1
24	299	0
24	261	0
48	381	0
48	262	0
48	324	0
96	252	2
96	211	0
96	109	0

Area = the area determined by "lanes" using SigmaGel

n = 10 for each sample

DISCUSSION

The expression of MS mRNA was studied in *C. riparius*. Chironomids were the organism of choice due to many reasons: 1) It is an EPA approved sediment teste organism (US EPA, 1996). 2) It is easy to handle in the lab (McCahone and Pascoe, 1988a). 3) It is the most abundant and diverse insect geographically (Taylor *et al.*, 1991). 4) It is the predominate animal found in sediments of most polluted ponds, lakes, and rivers (Yamamura *et al.*, 1983; Pinder, 1986). 5) Our laboratory is interested in studying diversity in chironomids population using MS mRNA. Studies conducted on other organisms such as mammals (Dib *et al.*, 1996), protozoa (Su and Willems, 1996), mosquitoes (de Sousa *et al.*, 1996; Lehmann *et al.*, 1996; Boyce *et al.*, 1997; Estoup *et al.*, 1996; Lanzaro *et al.*, 1995; Lehmann *et al.*, 1997; Schlottere *et al.*, 1997), and plants (Causse *et al.*, 1994) have used MS as a tool for genetic mapping. MS has high levels of polymorphism, and it is very abundant in genomes (Rongnoparut *et al.*, 1999). For these reasons, MS has been used for determining kinship (Queller *et al.*, 1993; Blouin *et al.*, 1996) and assessing intrapopulation variability and substructure (Bowcock *et al.*, 1994: England *et al.*, 1996; Favre *et al.*, 1997).

The sequences for MS cDNA and genomic DNA were obtained from a composite often larvae (Figure 1). The chironomid MS sequencing information was similar to MS sequences documented for other organisms (Colson and David B.G., 1999). The size of the MS band in *C. riparius* was within the expected range of 100 - 200 base pairs (Stephon and Cho, 1993). The sequence had 13 "ACA" repeats, 12 "AC" repeats, 5 "CTC" and 16 "CAA" repeats (Figure 2). This was comparable to other MS sequences in that it had repeats of dinucleotides and trinucleotides which were rich in adenine and cytosine (Colson and Goldstein, 1999; Griffith *et al.*, 1996). Using the MS primers, DD showed that there were

three bands produced that were close in size (Figure 3). All three bands were sequenced. Band A was identified as ribosomal mRNA, band B was unknown, and band C was identified as MS mRNA. Attempts were made to increase the stringency of the PCR condition by increasing primer annealing temperatures. These attempts significantly reduced the number of bands on DD. Therefore, the PCR cycle was modified in later experiments by increasing the annealing temperature from 54 °C to 57 °C.

Cadmium is a heavy metal that is a carcinogen. It has been found to induce expression of stress responsive genes (Liao and Freedman, 1998), including metallothionein in most organisms. The expression of the metallothionein gene was increased by 5.6 fold when Drosophila was exposed to 0.01 mM Cdat 24 h (Maroni *et al.*, 1986), and 15 fold when molluscs was exposed to 0.2 μM Cd at 24 h (Roesijadi *et al.*, 1994).

DD was used to determine if MS mRNA, in *C. riparius*, would respond to cadmium. Low and high cadmium concentrations were tested. Low cadmium concentration were 0.04, 0.4, and 4.0 mM (Figures 4 and 5). These concentrations were similar to the cadmium concentrations that cause metallothionein gene induction in *Drosophila melanogaster* (Maroni, 1995). The results from DD indicated that MS mRNA expression was not induced at low cadmium concentrations. Cadmium concentrations were increased to more lethal levels (1.0 mM, 2.0 mM, 4.0 mM, 8.0 mM, and 16.0 mM). The results from DD indicated that MS mRNA expression was comparable to control at these concentrations. The 24 and 48 h exposure times used in the low and high cadmium experiments were the same as those used in metallothionein gene induction in Drosophila (Maroni *et al.*, 1986) and molluscs (Roesijadi *et al.*, 1994).

The last Cd experiment, involving high cadmium toxicity, was conducted on fed larvae (Figure 7). The purpose of this experiment was to see if feeding would induce the level of MS mRNA expression. Research done by Postma (1994) and Pascoe (1990) has suggested that feeding increases cadmium toxicity. Feeding larvae during cadmium exposure might have increased, hypothetically, ingestion of cadmium as cadmium binds to food particles. However, the data did not show any induction of MS mRNA expression (Figure 7). In future studies, the feeding experiment should be repeated because certain factors were not taken into consideration. These factors, include the amount and kind of food given to the larvae and body weight, which plays a crucial role in cadmium exposure and should affect expression of stressed-genes as suggested by Lanno (1989) and Krantzber (1989).

There is no published data indicating the response of MS mRNA during acute cadmium exposure in other organisms. Based on our results it can be concluded that MS mRNA expression is unaffected by low or high cadmium concentrations in *C. riparius*. Additionally, feeding the larvae during the cadmium exposure made no difference. Therefore, MS mRNA can be used as an internal control for studying cadmium toxicity using other genes of *C. riparius*.

Many organisms have been found to respond to environmental stressors such as heat or chemical exposure by inducing certain genes (Bond *et al.*, 1993). For example, *Drosophila buskii* has a heat shock response when temperature is increased from 25 °C to 30 °C (Ritossa, 1962). Another study showed a heat shock response in *Drosophila melanogaster* when the temperature was increased from 20 °C to 28 °C and 32 °C (Govinda *et al.*, 2000). In order to see if MS mRNA would respond to elevated

temperatures in *C. riparius*, a heat shock experiment was performed. The larvae were exposed to 20 °C, 28 °C, 32 °C, or 37 °C for 30 min (Figures 8 and 9). The exposure period for heat shock response in *C. riparius* was similar to the exposure time used to cause a heat shock gene induction in Dorosophila (Govinda *et al.*, 2000). Results from DD indicated that MS mRNA expression was induced when exposed to 28 °C. However, when the temperature increased to 37 °C, the expression decreased. The change in expression suggested that the high levels of stress at 32 and 37 °C down regulated MS mRNA.

Since MS mRNA was significantly affected by heat shock, a control study was conducted. Previous work in *C. riparius* has shown that ribosomal protein mRNA expression is consistent at all of the temperatures tested (Govinda *et al.*, 2000). Therefore, ribosomal protein mRNA can be used as a control when studying heat shock in *C. riparius*. DD was run using the same mRNA as used for MS mRNA heat shock experiments (Figure 10). The level of ribosomal protein mRNA was unchanged by heat shock as expected. These results verify that the changes seen with MS mRNA were gene specific.

Studies done in our laboratory have indicated that *Chironomus* gene transcription is inhibited by ActD (Govinda *et al.*, 2000). ActD is an antibiotic that binds to DNA and causes a decrease in the level of transcription in all genes (Govidna *et al.*, 2000). As the concentration of ActD increases, the level of gene transcription should decrease. The level of MS mRNA expression was significantly reduced at the highest concentration of ActD, 96 nM (Figures 11 and 12).

During this project, the ability of DD to measure differentially expressed genes was evaluated. Some problems that were encountered are as followings: the bands were sometimes smeary and hard to distinguish, which could also be caused by many factors including poor RNA quality, old reagents, or gel conditions (Linskens *et al*, 1998). Also high background and intense bands made it difficult to read the results. Can DD be used to measure differentially expressed genes? Yes, the results presented here indicate that significant changes in gene expression can be measured. A modified DD technique has been developed, recently, called enhanced differential display (EDD; Linskens *et al*., 1998). EDD is more specific and gives reproducible results. In this method, the primers are allowed to bind nonspecifically for the first 10 cycles by reducing the optimal annealing temperature. For the remaining 20 cycles, the primer annealing temperature is increased to the optimal annealing temperature. This method reduces the number of bands produced in DD. In addition, certain things should be considered when performing DD. (1) RNA obtained should be subject to the Message Clean, which will degrade any protein and DNA contamination. (2) The reagents in the protocol should be fresh. (3) The running buffer should be made with the right pH and concentration. (4) The bands look better (less dark) when $\alpha[^{32}P]$ dATP is one week past its zero day. (5) Most importantly, the exact amount of each sample should be loaded on the gel. Any leakage of the sample in the buffer will result in inaccurate results. This skill is acquired with practice.

SUMMARY

The expression of MS mRNA was studied in *C. riparius*. The first part of the project involved sequencing a fragment of the MS gene from both the genomic DNA (Figure 1, lane 2) and cDNA (Figure 1, lane 3). The genomic MS band was~290 base pairs long and the cDNA MS band was ~260 base pairs long. Subsequently, the sequences showed that the size difference was probably due to a piece of intron at the 5' end. The cDNA MS band in DD was 168 bp, indicating that the positive primer was binding to more than one site along the MS gene. The sequencing information indicated 13 "ACA" repeats, 12 "AC" repeats, and 5 "CTC" repeats (Figure 2). The primers used during rPCRand PCR were MSM25 and MSP5 (Table 1). The primer annealing temperature was modified from 54 °C to 57 °C, which gave more specific products on DD. The primers generated multiple bands only one of which was MS mRNA. DD allowed us to quanitate the MS mRNA in exclusion of the other two.

The second part of this project was to study the effects of Cd on MS mRNA expression in *C. riparius* using DD. Experiments above sbowed that when larvae were exposed to low Cd concentrations for 24 b, the expression of MS mRNA was not induced ($p = 0.732$). Increasing the Cd exposure period to 48 b sbowed that the level of MS mRNA was still unaffected ($p = 0.885$). However, as the Cd exposure period increased by a day, the mortality rate increased by one fold (Table 2). Since, exposure to low Cd concentration at 48 h did not show induction of MS mRNA, the Cd concentration was increased to a more lethal dose (16 mM-high Cd concentration). Results clearly indicated that the level of MS mRNA expression was not affected at high Cd concentration ($p = 0.702$). However, the mortality rate increased drastically (Table 2). The last experiment,

involving high Cd toxicity at 48 h, was conducted on larvae of *C. riparius* that were fed. Results showed that MS mRNA was not induced in the fed larvae ($p = 0.857$). Overall results showed that MS mRNA was not affected by any Cd concentrations tested in fed or starved larvae. Thus, MS mRNA can be used as an internal control for studying Cd toxicity in other genes of *C. riparius*.

The third part of the project involved exposing *C. riparius* to different temperatures. The heat exposure period was 30 min. Results showed that the level of MS mRNA expression was significantly decreased at 37 °C compared to control ($p = 0.002$). The level of MS mRNA was significantly increased when the temperature was elevated from 20°C to 28°C ($p = 0.033$). In addition, the 32 and 37 °C groups were significantly lower than the 28°C group ($p = 0.014$ and 0.002, respectively.)

Since the effects of temperatures were significant, a control study was conducted. When studying gene expression in *C. riparius*, ribosomal L8 mRNA is used as an internal control because it is not induced by Cd or heat shock (Govinda *et al.*, 2000). The mRNA from the heat shock experiment was reverse transcribed and amplified using specific primers for ribosomal L8 mRNA. Results showed no induction of ribosomal L8 mRNA, as expected ($p = 0.173$). This indicates that the changes in MS mRNA in heat shock studies were gene specific.

In the last part of this project, ActD was used to show the down regulation of MS mRNA. ActD is a chemical that binds to DNA and causes a decrease in the level of transcription in all genes. As the concentration of ActD increases the level of gene transcription should decrease (Govinda *et al.*, 2000). The level of MS mRNA expression was significantly reduced at the highest concentration of ActD, 96 nM ($p = 0.054$).

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